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Isolation and Biochemical Characterization of the Human Dkk-1 Homologue, a Novel Inhibitor of Mammalian Wnt Signaling*

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In an effort to isolate novel growth factors, we identified a human protein, designated Sk, that co-eluted with Neuregulin during chromatographic separation of conditioned medium from the SK-LMS-1 human leiomyosarcoma cell line. Degenerate oligonucleotides based on amino-terminal sequence analysis of the purified protein were used to isolate the corresponding cDNA from a library generated from this cell line. Sk is a novel 266-amino acid protein that contains a signal peptide sequence and two cysteine-rich domains with no similarity to other known growth factors. A single major 2-kilobase transcript was expressed in several embryonic tissues. Transfection of mammalian cells demonstrated that the protein was secreted and expressed as a doublet of approximately 35 kDa. *In vitro* translation and endoglycosylase analysis indicated that this doublet, which was also observed in cells expressing the endogenous protein, arises from posttranslational modification. A search of the GenBank™ data base revealed a match of Sk with Dkk-1, which is a novel secreted protein required for head induction in amphibian embryos and a potent Wnt inhibitor. When coexpressed with Wnt-2 in NIH3T3 cells, human Sk/Dkk-1 caused reversion of Wnt-2 induced morphological alterations and inhibited the Wnt-2 induced increase in uncomplexed β-catenin levels. These results provide biochemical evidence that human Sk/Dkk-1 antagonizes Wnt signaling upstream of its effect on β-catenin regulation.

Members of the Wnt gene family encode secreted glycoproteins that are required for a variety of developmental processes ranging from cell lineage decisions to control of differentiation of the central nervous system in higher vertebrates (1, 2). In *Xenopus* embryonic development, Wnt signaling is involved in dorsoventral axial patterning both before and after the midblastula transition (1). Wnts bind and act through the frizzled family of cell surface proteins, a large family of seven membrane-spanning domain receptors (3). A number of downstream components of Wnt signaling have been identified by a combination of genetic and biochemical approaches. Wnts act through the cytoplasmic protein dishevelled to inhibit the activity of the serine threonine kinase glycogen synthetase kinase-3. Glycogen synthetase kinase-3 appears to bind through a bridging molecule, Axin, to the β-catenin/adenomatous polyposis coli complex and phosphorylate β-catenin, causing its rapid degradation. Wnt-induced inhibition of glycogen synthetase kinase-3 leads to β-catenin stabilization resulting in an increased level of the uncomplexed soluble form (4–6). The latter can interact with T-cell factor/LEF transcription factors and, after translocation to the nucleus, activate target genes (7).

There is evidence that activation of Wnt signaling can contribute to the neoplastic process. Inappropriate expression of these ligands due to promoter insertion of the mouse mammary tumor virus (reviewed in Ref. 2) or targeted expression in transgenic mice causes mammary tumor formation (8). Moreover, in cell culture, several Wnt family members have been shown to induce altered morphology and increased saturation density of certain epithelial (9, 10) and fibroblast (11, 12) cell lines. Finally, genetic alterations affecting adenomatous polyposis coli or β-catenin levels, have been observed in human colon cancer (13), melanomas (14), and hepatocellular carcinomas (15), indicating that aberrations of Wnt signaling pathways are critical to the development of these and possibly other human cancers.

Recent studies in *Xenopus* embryos have identified several families of inhibitors of Wnt signaling. One, designated as frizzled-related protein (FRP)[1] (16), has structural similarities to the frizzled family of Wnt receptors. Wnt interacts with frizzled to induce axis duplication following coinjection of early *Xenopus* embryos (17). In contrast, FRP, also designated as Frzb (18, 19), inhibits Wnt-induced axis duplication. FRP shares with frizzled a cysteine-rich domain, which has been shown to confer Wnt binding properties (3). Thus, FRPs presumably act as Wnt antagonists at the level of receptor binding.

The gene *cerberus* encodes for a secreted protein that is capable of inducing ectopic head formation in *Xenopus* embryos (20). It has been recently shown that this protein is a multivalent growth factor antagonist capable of binding Nodal, bone morphogenetic protein, and Wnt proteins, inhibiting their effects (21).

Another inhibitor was isolated by an expression cloning strategy for cDNAs able to complement in the formation of a complete secondary axis in *Xenopus* (22). A novel molecule, designated dickkopf-1 (dkk-1) (German for "big head, stub-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF127563.

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[1] The abbreviations used are: FRP, frizzled-related protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; kb, kilobase.

born") encoded by the isolated cDNA, contained a signal sequence as well as two cysteine-rich domains and was unrelated to any previous protein. Like FRP, Dkk-1 was shown to possess the ability to inhibit Wnt-induced axis duplication. Genetic analysis indicated further that Dkk-1 acts upstream of dishevelled to inhibit Wnt signaling (22). In the course of efforts to isolate novel epidermal growth factor-like ligands, we identified a human Dkk-1 homologue, which we have characterized in the present study with respect to its expression pattern and function as an inhibitor of Wnt signaling in mammalian cells.

EXPERIMENTAL PROCEDURES

Purification, Physical Characterization, and Microsequencing—SK-LMS-1 cells were plated onto 175-cm^2 T flasks and grown to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (v/v) calf serum (Life Technologies, Inc.). Conditioned medium was then generated by two consecutive harvests in serum-free medium for 72 h, alternate by 4 days of culturing with serum containing medium. Cells were washed twice with phosphate-buffered saline (PBS) prior to each harvest cycle. Approximately 5 liters of pooled conditioned medium was filtered through a 0.45-μm cellulose acetate membrane (Corning) and concentrated 40 times by an Amicon ultrafiltration system, using membrane of 20-kDa molecular size cut-off. Purification steps were performed by means of a Bio-Rad Econo low pressure chromatography system. Aliquots from each step were diluted in Dulbecco's modified Eagle's medium and tested for their capacity to induce erbB3 tyrosine phosphorylation as described previously (23). Silver staining (Amersham Pharmacia Biotech) was used to monitor the level of purification through different steps. The concentrated material was directly loaded on a column of heparin-Sepharose (5 ml, HiTrap, Amersham Pharmacia Biotech). The column was washed twice with PBS to remove nonspecifically bound proteins. Elution was performed by a step gradient of NaCl (0.2, 0.4, 0.6, 0.8, 1, and 2 M) and 10-ml fractions were collected. The 1 M active fraction was diluted to adjust the NaCl concentration to 0.15 M, and applied to a cation exchange column (1 ml, HiTrap SP, Amersham Pharmacia Biotech). Bound proteins were then eluted with a step gradient of NaCl (0.2, 0.4, 0.6, 0.8 and 1 M), and 3-ml fractions were collected. The active 0.4 M fraction was then subjected to separation using a Cu^{2+} chelating column (1 ml, HiTrap, Amersham Pharmacia Biotech). Competitive elution was performed by a linear gradient of NH_4Cl (from 0.4 to 1 M) in 0.02 M Na_2PO_4 at pH 7.4. Silver staining analysis of the active fractions revealed a 35-kDa doublet. Such samples were combined, quantitated, and subjected to microsequencing. Approximately 3 μg of protein was separated by SDS-PAGE, transferred to an Immobilon membrane and loaded into an Applied Biosystem gas-phase protein sequenator, equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A, Applied Biosystems).

Molecular Cloning and Analysis—Poly(A)$^+$ RNA was isolated from SK-LMS-1 cells by oligo(dT) cellulose column chromatography (Collaborative Research, Type 3) and a cDNA library constructed as described previously (24, 25). For isolation of the cDNA encoding the microsequenced protein, nested PCR amplifications were conducted on the cDNA library phages, using vector-specific primers upstream (*Bam*HI-adapted) and degenerate primers corresponding to amino-terminal protein sequence downstream (*Eco*RI-adapted). In an attempt to reduce degeneracy of gene-specific primers, inosine (I) residues were incorporated. For the first PCR amplification, the oligonucleotides 5′-CGGGATCCGATCATTTAGGTGACACACTATA-3′ and 5′-GGAATTCAC(G/A)GC(G/A)TCICCIGG(G/A)TG-3′ were used with the cDNA library as template. The following nested PCR was performed by using as template an aliquot of the first PCR reaction and 5′-GAATACGGATCCGTCGACGGC-3′ and 5′-GGAATTCGGIAG(A/G)TT(C/T)TT(G/A/T)ATIGC(G/A)TT-3′ as primers. Southern blot hybridization was used to monitor specificity of the correct amplification product using the degenerate oligonucleotide 5′-GGIA(G/A)(G/A)TT(C/T)TTIATIGC(G/A)TTI(G/C)(A/T)(G/A)TT-3′ as a probe. Following cloning of the PCR product in the TA cloning vector (Stratagene), positive clones were identified by hybridization and subjected to sequence analysis by dideoxy chain-termination. A gene-specific insert was used as a probe for screening of the SK-LMS-1 cDNA library to isolate the entire coding sequence as described previously (26). Ten positive clones were isolated and classified into three groups based on the restriction pattern after digestion with *Not*I. The insert from one representative clone from each group was sequenced on both strands using the ABI373A automated DNA sequencer and the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc.). Search for homology with known sequences was performed using the National Center for Biotechnology data base (http://www.ncbi.nlm.nih.gov). Alignments were generated with the Pile-up and Bestfit programs from the Wisconsin Package, Version 9.1 (Genetics Computer Group, Madison, WI).

Antiserum Production—To generate antiserum to the protein, a *Sal*I site was generated by PCR in frame with the coding sequence and subcloned in the pGEX-5X-2 vector (Amersham Pharmacia Biotech). The bacterially induced GST fusion protein was subjected to SDS-PAGE followed by electroelution. Immunization of rabbits was performed as described previously (27)

Northern Blot Analysis—Total RNA was isolated by using RNAZol (Tel-Test, Inc.). RNA samples (25 μg) were separated by denaturing agarose gel electrophoresis and transferred to a nitrocellulose filter, followed by hybridization with ^{32}P-labeled probe as described (26, 28). Northern blots of poly(A)$^+$ RNA isolated from a variety of adult and embryonic tissues were purchased from CLONTECH and hybridized following manufacturer's specifications.

Cell Lines and Transfections—The human tumor cell lines SK-LMS-1, SK-OV3, and PA-1, were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

For transfection analysis, the cDNA was subcloned in pCEV29, after removal by restriction digestion of 5′- and 3′-untranslated regions using the restriction enzymes *Ple*I and *Bsm*I, respectively. The Wnt-2 HFc fusion protein construct has been previously described (11).

NIH3T3 cells were plated at 1.5×10^5 per plate in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Life Technologies, Inc.). After 24 h, cultures were transfected with 1 μg of each plasmid DNA by the calcium phosphate method, as described previously (29). Marker selected cultures were obtained by addition to the growth medium of 750 μg/ml of Geneticin (Life Technologies, Inc.) and 2 μg/ml of puromycin (Calbiochem).

Subconfluent cultures of 293T cells (11) were subjected to transfection by the calcium phosphate method with 3 μg of plasmid DNA. Cultures were incubated with the precipitated DNA for 5–6 h and then washed in growth medium. Twenty-four hours later, cells were washed and incubated in serum-free medium, and conditioned medium was collected after an additional 24 hours.

Protein Analysis and in Vitro Transcription and Translation—Aliquots (1 ml) of conditioned medium harvested from tumor cell lines or transfected cells, were subjected to immunoprecipitation followed by immunoblotting analysis as described previously (27). Incubation was carried at 4 °C for 1 h, followed by addition of Gammabind G-Sepharose (Amersham Pharmacia Biotech). Immunoprecipitates were washed three times with PBS containing 1% Triton, 0.1% SDS, and 0.5% sodium deoxycholate, and separated by SDS-PAGE (29). Immunodetection was then performed by use of ^{125}I-labeled protein A. *In vitro* transcription and translation was performed with a commercially available kit from Promega (TNT™ coupled reticulocyte lysate system). Briefly, 0.5 μg of cDNA was used for each reaction with or without the addition of canine pancreatic microsomal membrane (Promega), following the manufacturer's instruction. Translation products were separated by SDS-PAGE and subjected to autoradiography. ^{14}C-labeled marker was used for molecular weight determination. For Wnt-2 HFc detection, 100 μg of total cell lysates from cultures solubilized in radioimmune precipitation buffer were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. Specific signals were visualized by ECL analysis (Amersham Pharmacia Biotech) after incubation with horse peroxidase conjugated rabbit anti-mouse HFc antibody (2.6 μg/ml; DAKO).

Enzymatic Deglycosylation—One ml of conditioned medium from cultured SK-LMS-1 cells was immunoprecipitated using the rabbit antiserum as described previously. Immunoprecipitated proteins were resuspended in 40 μl of PBS that contained 0.1% SDS. Samples were then boiled for 5 min, followed by addition of 100 μl of PBS and Nonidet P-40 (final 1%). *N*-GlycosydaseF (20 units, Roche Molecular Biochemicals) was added, and samples were incubated at overnight 37 °C The digested proteins were then separated on a 12% acrylamide SDS-PAGE and subjected to immunoblotting analysis with Sk antiserum.

GST-E-cadherin Binding Assay—The GST-E-cadherin binding assay was performed as described previously (11). Briefly, bacterially expressed GST-E-cadherin was purified with glutathione-Sepharose beads and incubated with 0.1 or 1 mg of each cell lysate. The GST-E-cadherin/β-catenin complex bound to the beads was recovered by centrifugation and analyzed by SDS-PAGE followed by immunoblotting with the anti-β-catenin antisera (Transduction Laboratories). Detection was performed with ^{125}I-labeled protein A.

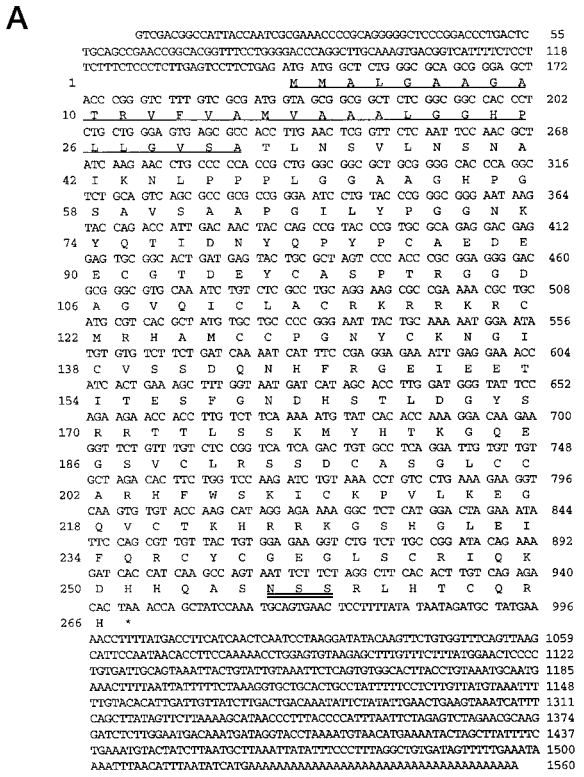

FIG. 1. *A,* nucleotide and deduced amino acid sequences of Sk cDNA. The signal peptide is *underlined*. A potential site of *N*-glycosylation is *double underlined*. Numbers represent nucleotide sequence on the right and amino acid sequence on the *left*. *B,* hydrophilicity profile of the Sk protein sequence determined by the Kyte and Doolittle method (31).

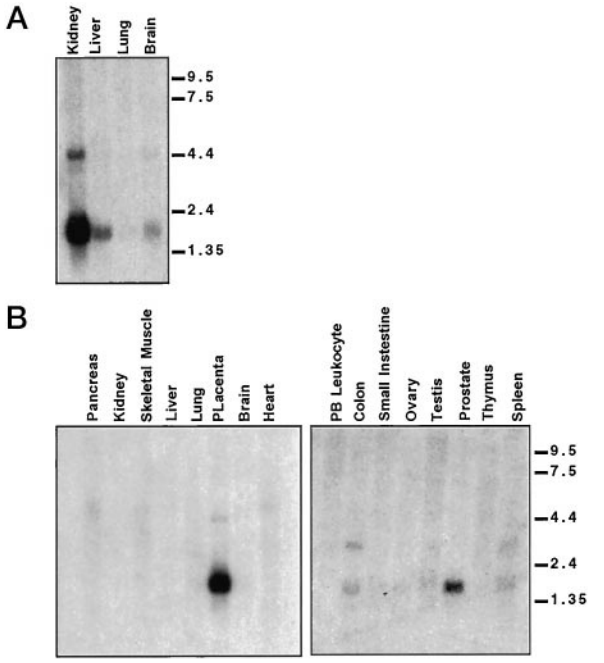

FIG. 2. **Expression of human Sk/Dkk-1 in fetal and adult human tissues.** Commercially available blots (CLONTECH) with approximately 2 μg of poly(A)$^+$ RNA from different tissues (fetal (*A*) and adult (*B*) tissues) were hybridized using full-length Sk cDNA as probe. *PB,* peripheral blood.

RESULTS

Purification and Molecular Cloning of a Novel Human Secreted Molecules—In the course of the purification of ligands for the erbB3 receptor, we identified a protein that appeared as a doublet of 35 kDa by silver staining analysis and co-eluted with Neuregulin. Microsequencing of the purified material revealed positive identification for both bands in a single run of several amino acids as follows: TLNSVLNSNAIKNLPPPLGGAAGH-PGDAV*X*A (*X* indicates inability to make an amino acid assignment). Comparison of the amino-terminal sequence of the purified protein with those in several data bases revealed no significant homology, suggesting that this might be a novel protein.

Degenerate oligos were designed for isolation by PCR of a gene-specific insert that was used for the cloning of the full-length coding sequence from a SK-LMS-1 cDNA library. After three consecutive screening cycles, positive clones were classified into three groups based on their restriction digestion patterns. Sequence analysis of one clone (1.5 kb) demonstrated an open reading frame of 798 base pairs surrounded by 145 base pairs of 5′ noncoding sequence and 614 of 3′ noncoding sequence (Fig. 1*A*). The 3′ noncoding region contains a polyadenylation signal, a mRNA instability motif as well as a poly(A) tail. Two consecutive ATGs, starting at nucleotides 146 and 149, were identified as initiation codons, fulfilling the consensus sequence for a translation initiation codon as defined by Kozak (30). Two upstream in-frame stop codons were also identified. The open reading frame predicted a 266-amino acid protein with an approximately 26-kDa molecular mass and a isoelectric point of 9.67 determined by using the MacVector program (Eastman Kodak). The experimentally determined protein sequence began 32 residues downstream of the first identified methionine. Hydrophilicity analysis (31) of the coding sequence revealed a hydrophobic region at the amino terminus, 31 amino acids upstream of the experimentally determined sequence, which likely functioned as signal peptide for protein secretion (Fig. 1*B*). Several cysteine residues, clustered into two separate areas, were also identified in the amino acid sequence, consistent with the secreted nature of the protein. However, no similarity was detected with the epidermal growth factor-like family of growth factor or other known growth factors or secreted proteins. A potential site for *N*-linked glycosylation in the carboxyl-terminal region of the protein was identified at position 256–258, suggesting posttranslational modification. Consistent with this possibility was the size of the isolated doublet, which was larger than the predicted molecular weight. The other clones (2.5 and 1.2 kb) contained the same coding sequence but differed in size from the 1.5-kb clone due to variations in their 3′-untranslated regions. The 2.5-kb clone also contained an unspliced intron.

Pattern of Human Sk Tissue Expression—We performed Northern blot analysis to determine expression of Sk in different human tissues using the entire coding sequence as probe. In a panel of poly(A)$^+$ mRNAs from fetal tissues, a strong 2-kb transcript was detected in kidney. A less intense band was present also in liver and brain but not in lung (Fig. 2*A*). In adult tissues, a similar size transcript was identified in placenta and prostate (Fig. 2*B*). Hybridization signals were also visible after longer exposure in colon and spleen. A lower level of expression of a higher molecular weight transcript was also

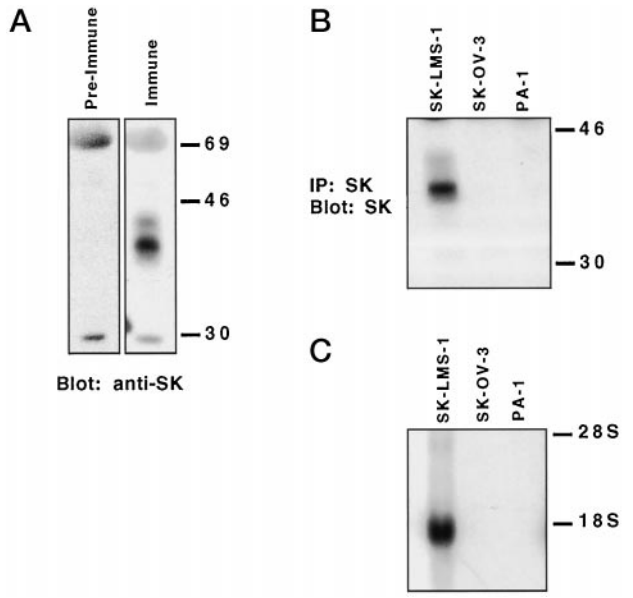

FIG. 3. **Characterization of the human Sk protein.** *A,* Western blot analysis using serum from a rabbit immunized with a GST-Sk fusion protein. The antiserum specifically recognized a protein in concentrated conditioned medium from SK-LMS-1 cells. *B,* immunoprecipitation and Western blot analysis of Sk protein in conditioned medium from various human tumor cell lines. The antiserum specifically recognized an immunoreactive 35-kDa protein in conditioned medium from SK-LMS-1 but not from SK-OV-3 or PA-1 cells. The positions of molecular weight markers are indicated at the *right*. *C,* Northern blot analysis of the Sk transcript in tumor cell lines. 30 μg of total RNA was probed with the human Sk cDNA probe. The positions of 28 S and 18 S ribosomal RNA are shown.

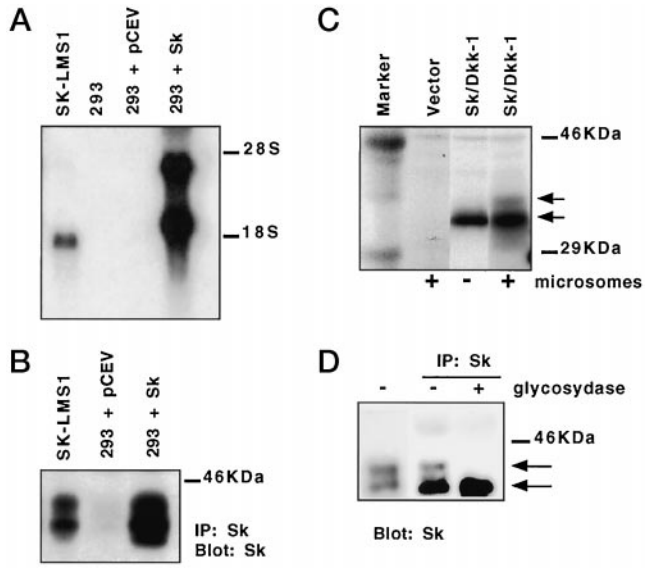

FIG. 4. **Characterization of the human Sk protein in transfected cells.** Sk cDNA was subcloned in a mammalian expression vector (pCEV29) and transiently transfected in 293 cells. *A,* Northern blot analysis using 30 μg of total RNA from transiently transfected cells probed with human Sk cDNA. *B,* approximately 48 h following transfection, 1 ml of conditioned medium was collected and subjected to immunoprecipitation followed by immunoblot analysis with anti-Sk serum. Conditioned medium from SK-LMS-1 cells was used as control. *C, in vitro* transcription and translation of human Sk cDNA. 1 μg of plasmid was subjected to *in vitro* transcription/translation using a coupled reticulocyte lysate system (Promega). [^{35}S]methionine-labeled species are identified by an *arrow* and a *asterisk*. Canine microsomal membranes were added as indicated. Molecular weight markers are shown on the *right*. *D,* deglycosylation of SK/Dkk-1. Conditioned media from SK-LMS-1 cells were immunoprecipitated and subjected to enzymatic treatment with *N*-GlycosydaseF overnight at 37 °C. Digested samples were separated on a 12% SDS-PAGE and subjected to immunoblotting with Sk antiserum. 100 μl of conditioned medium was loaded at *left* as control. *Arrows* at *right* indicate the two Sk/Dkk-1 isoforms.

detected both in embryonic and adult tissues. It is conceivable that these larger transcripts may represent signals from unprocessed mRNA. However, we cannot exclude the possibility that they may reflect cross-hybridization with transcripts of related proteins.

Identification and Characterization of the Human Sk Protein—A polyclonal antibody was generated against the full-length human Sk protein, produced as a GST fusion molecule in *E. coli*. Immunization was monitored by testing the capacity of antisera obtained from successive bleeds to recognize the GST fusion protein and the endogenous molecule in concentrated conditioned medium from SK-LMS-1 cells. As shown in Fig. 3*A,* the antiserum specifically recognized a major species of approximately 35 kDa in conditioned medium of SK-LMS-1 cells. Moreover, the protein was also detected by immunoprecipitation followed by immunoblotting with the same antiserum (Fig. 3*B*). Therefore, the polyclonal antibody generated was capable of identifying the endogenous Sk protein in a native as well as denatured state. A less intense immunoreactive species migrating more slowly was detected under both conditions. In contrast, the same antiserum failed to detect a similar band in culture fluids of SK-OV-3 or PA-1 cells, which lacked any detectable Sk transcript (Fig. 3, *B* and *C*).

To further confirm that the cDNA isolated encoded for the expected protein, we transfected 293 cells with the human Sk gene after removing its 5′- and 3′-untranslated regions and subcloning in pCEV 29 vector (25). Northern blot analysis was first performed in such cells to determine transfection efficiency. Fig. 4*A* shows that the levels of Sk transcripts in transfected 293 cells were much higher than present in SK-LMS-1 cells and appeared as two specific bands that migrated more slowly than the endogenous 2-kb transcript present in SK-LMS-1. To correlate RNA levels with protein expression, conditioned medium from transiently transfected cells was analyzed by immunoblotting for the presence of Sk immunoreactive protein. As shown in Fig. 4*B,* immunoreactive bands in the conditioned medium of Sk transfected 293 cells showed similar sizes to two species identified in SK-LMS-1 medium, and were not present in mock transfectants. The stronger signal identified in 293 cells correlates well with the higher transcript levels shown by Northern analysis.

Our findings that the doublet expressed in 293 cells was encoded by a single cDNA, strongly supported the possibility that post translational modification was responsible for these two immunoreactive species. To test this hypothesis, we performed an *in vitro* transcription and translation analysis. As shown in Fig. 4*C,* a single radiolabeled protein of approximately 35-kDa was observed in the absence of microsomal membranes. In their presence, an additional less intense band of slightly higher molecular weight became evident as well. These species resembled the appearance of the two immunoreactive bands in conditioned medium of SK-LMS-1 or 293 transfectants cells, consistent with the conclusion that Sk undergoes posttranslational modification. Because a potential site for *N*-linked glycosylation is present in the carboxyl-terminal region of the protein we tested whether glycosylation was indeed responsible for the appearance of the doublet. Sk was immunoprecipitated from conditioned medium and subjected to enzymatic deglycosylation using *N*-glycosidaseF, which releases all types of asparagine bound *N*-glycans, as described under "Experimental Procedures." This analysis (Fig. 4*D*) revealed that after treatment, Sk appeared as a single major immunoreactive band of similar molecular weight to the lower species

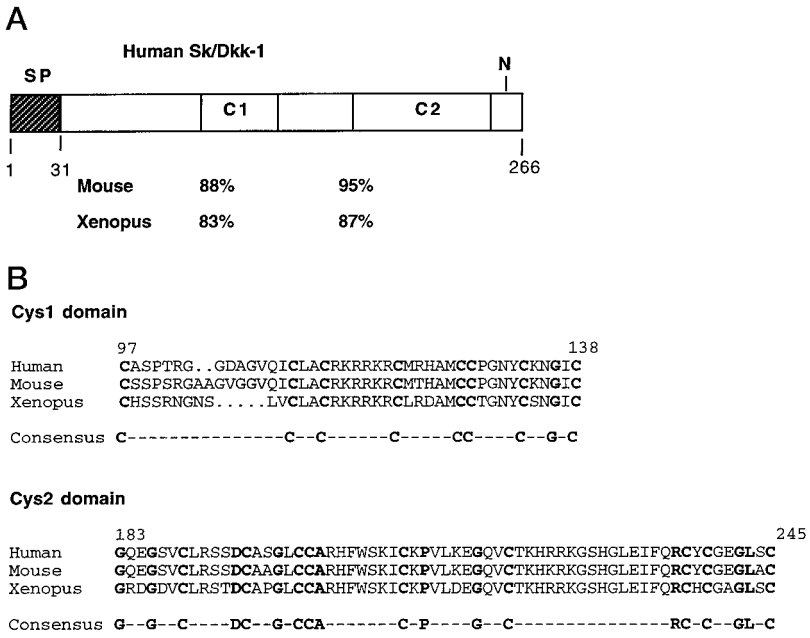

FIG. 5. *A,* schematic representation of the Sk/Dkk-1 protein. *SP,* signal peptide; *C1,* cysteine 1 domain; *C2,* cysteine 2 domain; *N, N*-glycosylation site. Percentage of identity between human and mouse or *Xenopus* cysteine-rich domains is shown. *B,* pile-up of Dkk-1 sequences from human (AF127563), mouse (AF030433), and *Xenopus* (AF030434) proteins in the two cysteine-rich domains. The consensus sequence is as reported by Glinka *et al.* (22).

in the untreated control. These results demonstrate that *N*-glycosylation is responsible for the higher molecular weight species.

Although our discovery of Sk was based on the co-elution with neuregulin during chromatographic purification, subsequent experiments with the recombinant protein, indicated that Sk was not a ligand for erbB molecules (data not shown), consistent with the absence of any similarity with epidermal growth factor-like proteins. Due to the novel features of the Sk protein, we continued to search the GenBank™ data base for related molecules in an effort to obtain insights into Sk biological function. In 1997, significant similarity was observed with a protein of unknown function identified in chicken (GenBank™ accession number 517093). A more recent report of a novel secreted protein from the Spemann organizer in amphibian embryos (22) was instrumental in identifying Sk function.

SK Is the Human Homologue of Dickkopf-1—Dickkopf-1 (*dkk-1*) was identified as a secreted protein that is required for head formation during *Xenopous* embryogenesis (22). By search of the expressed sequence tag-sequence data base, and a mouse cDNA library screening, it was further shown that *Xenopous dkk-1* is a member of a new family of genes, composed of at least three members in humans, and present in different species (22). Comparison of the Sk sequence with the *dkk-1* gene, revealed that Sk was the human homologue of *dkk-1*. In fact, Sk was identical in the region of overlap with the human *dkk-1* gene, identified as partial clone in the expressed sequence tag-sequence data base (GenBank™ accession number AA207078).

Moreover, the predicted Sk protein possessed all of the structural featured described for Dkk-1. In particular, it showed a perfect consensus for the two highly cysteine-rich regions (C1 and C2 domains) conserved in this family (22). Comparison of the amino acid sequence of Sk revealed 88 and 83% identity with mouse and *Xenopus* Dkk-1 C1 domains, respectively, and 95 and 87% amino acid identity in C2 domains (Fig. 5). The high percentage of identity among different species in these two regions predicts a highly conserved functions for these domains. In particular, it has been reported that the spacing of cysteine residues in the C2 domain resembles the colipase fold pattern, suggestive of possible lipid interaction of this domain (32). Lower levels of homology were identified for the other protein domains with the exception of the region located between the signal peptide and C1. In fact, this domain showed a 89% identity between human and mouse and 49% relatedness to the *Xenopus* protein. Moreover, in the carboxyl-terminal region, the last nine amino acids were identical in all three sequences analyzed. Thus, Sk was the human homologue of Dkk-1.

Human SK/Dkk-1 Inhibits Wnt-induced Morphological Transformation and Signaling—It has been previously shown that injection of mRNA encoding Dkk-1 in *Xenopus* embryos is sufficient and necessary to cause head induction (22). This effect seems to be mediated by the ability of Dkk-1 to antagonize Wnt signaling, as demonstrated by the ability of *dkk-1* to rescue the secondary-axis duplication induced by *Xwnt-8* (22). To assess the ability of human Sk to function as a Wnt inhibitor in a mammalian system, we transfected NIH3T3 cells with human Wnt-2 and human Sk/Dkk-1, individually or in combination, to determine the capacity of Sk/Dkk-1 to revert Wnt-2-induced alterations in the growth of these cells (11). Stable transfectants were obtained by double marker selection and grown postconfluence. As shown in Fig. 6, and quantitated in Table I, Wnt-2 expressing cells grew to higher cell density then the vector control. Coexpression of Sk/Dkk-1 with Wnt-2 essentially blocked this effect such that human Dkk-1/Wnt-2 cotransfectants were indistinguishable from the controls.

Evidence indicates that Wnts act through dishevelled to regulate β-catenin stability (9). In *Xenopus*, genetic analysis has indicated that Dkk-1 inhibits Wnt signaling upstream of dishevelled (33). The morphologic effects of transforming Wnts in mammalian cells such as NIH3T3 are associated with an increase in β-catenin stability as measured by the level of uncomplexed β-catenin using a GST-E cadherin binding assay (11). To assess the effects of human Sk/Dkk-1 expression on Wnt-2 signaling in NIH3T3 cells, we quantitated the levels of uncomplexed β-catenin in Wnt-2 and Wnt-2/Sk/Dkk-1 transfectants. As shown in Fig. 7*A,* Sk/Dkk-1 coexpression drastically inhibited the Wnt-2-induced increase in uncomplexed β-catenin. Comparable levels of Wnt-2 expression were observed in those cells, excluding the possibility that Sk activity was due to inhibition, direct or indirect, of Wnt expression (Fig. 7*B*). These results demonstrate biochemically that human Sk/Dkk-1 interferes with Wnt signaling and acts at a level upstream of β-catenin stabilization.

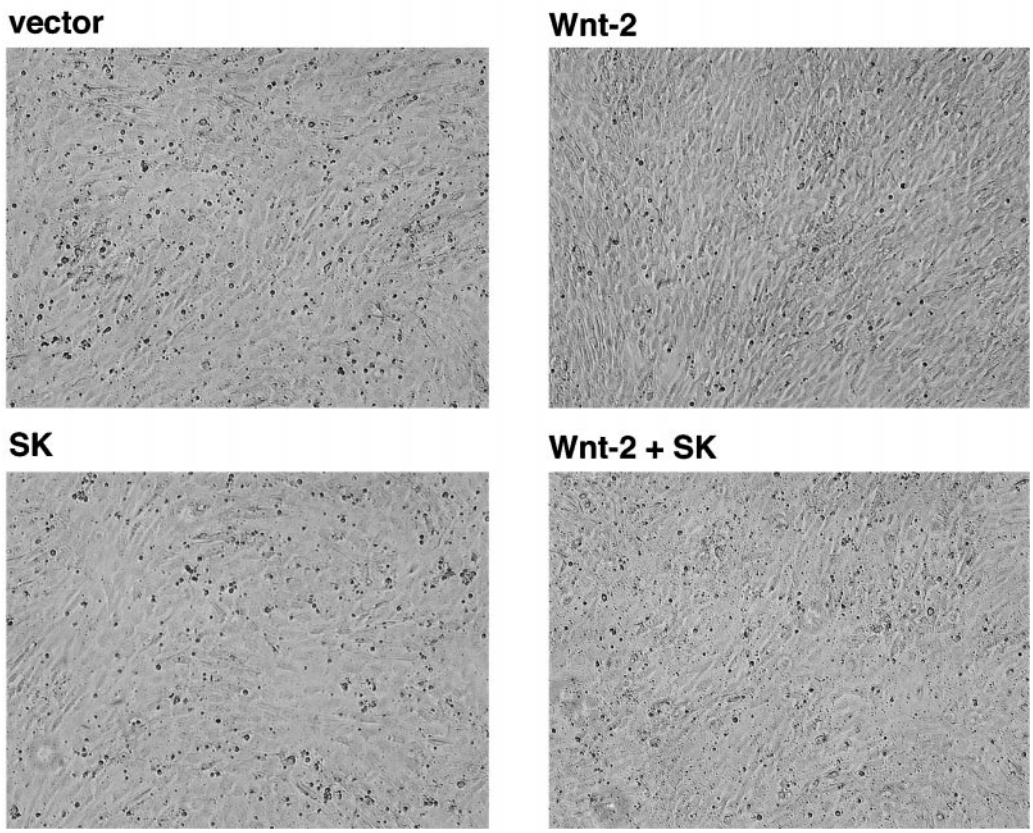

FIG. 6. **Human Sk/Dkk-1 inhibits Wnt-induced morphological transformation in NIH3T3 cells.** Cells were cotransfected with pBabe puromycin and pCEV29-neomycin vector, pBabe-Wnt-2-puromycin and pCEV29-neomycin, pBabe-puromycin and pCEV29Sk-neomycin or pBabeWnt-2-puromycin and pCEV29Sk-neomycin. Double marker selected cells were grown postconfluence and photographed.

TABLE I
Saturation density of NIH 3T3 transfectants

1 μg of each plasmid DNA was used for transfection. Cells from marker selected cultures were counted after 2 weeks.

NIH 3T3 transfectant	No. of cells/100-mm plate ($\times 10^{-6}$)
Vector	6.4 ± 0.5
Wnt-2	17.4 ± 1.4
Wnt-2 + SK	8.2 ± 0.6
SK	6.0 ± 0.4

DISCUSSION

In this report we describe the identification and characterization of a novel human protein Sk, isolated as an heparin-binding protein that co-purified with Neuregulin in conditioned medium from the SK-LMS-1 human leiomyosarcoma cell line. A continued search of the GenBank™ data base for related molecules revealed a sequence match with the *Xenopus* Dkk-1 protein recently reported (22). Dkk-1 was identified by an expression cloning strategy in an effort to identify novel genes that function as head inducers in *Xenopus* embryo development (22). Co-injection experiments also revealed that Dkk-1 is a potent inhibitor of Wnt signaling, as indicated by its ability to completely rescue the formation of a secondary axis induced by *XWnt-8*. These findings encouraged us to investigate human Sk/Dkk function with respect to Wnt signaling in mammalian cells. We demonstrated that Sk/Dkk, when coexpressed in NIH 3T3 cells, is able to cause reversion of Wnt-induced morphological transformation. Moreover, this inhibition correlated with its ability to cause a dramatic decrease in Wnt-induced accumulation of uncomplexed β-catenin. These biochemical findings complement genetic analysis in *Xenopus* indicating that Sk/Dkk-1 acts upstream of dishevelled to inhibit Wnt signaling (22).

The endogenously expressed human Sk/Dkk-1 protein appeared as a secreted doublet of approximately 35 kDa on SDS-PAGE either after chromatographic purification or when detected by immunoblot in conditioned medium. Two similar sized species were identified in transfected 293 cells, implying that a single cDNA encoded both forms. In *Xenopus*, exogenous Dkk-1 was expressed as two species, of which the higher molecular weight form was predominantly secreted (22). All of these findings suggest that this molecule undergoes posttranslational modification. Our *in vitro* transcription and translation analysis of Sk cDNA proved that in addition to a lower molecular weight species of around 35 kDa, a slower migrating protein was synthesized in the presence of microsomal membranes, consistent with the latter arising from posttranslational modification. Enzymatic deglycosylation of immunopurified Sk/Dkk-1 from conditioned medium of SK-LMS-1 cells, confirmed that the higher molecular weight species represented the *N*-glycosylated form of the protein.

Northern blot analysis revealed that Sk/Dkk-1 is broadly expressed in embryonic tissues. The presence of the Sk transcript in human fetal kidney, liver, and lung suggests that Sk may play a role in the development of these organs, consistent with the reported expression of Dkk-1 in foregut endoderm of mouse embryos (22). The limited expression of Sk detected in several human adult tissues does not exclude a possible role of this protein in later development/differentiation. However, more sensitive methods of analysis will be required to establish whether expression can be demonstrated in specific cell types. Moreover, because Sk was isolated as a secreted molecule from a tumor cell line, analysis for Sk expression in tumor tissues

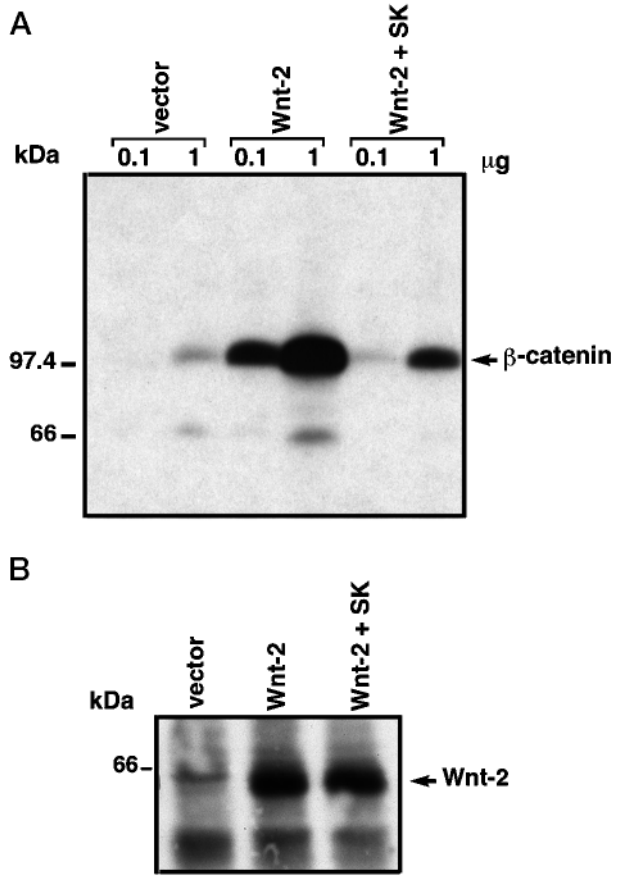

FIG. 7. **Human Sk/Dkk-1 antagonizes Wnt-2 signaling in NIH3T3 cells.** *A,* stable transfectants were lysed, and either 0.1 or 1 mg of total lysates was analyzed for uncomplexed β-catenin levels as previously reported (11). *B,* Western blot analysis of Wnt-2 expression in NIH3T3 transfectant cells. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting with a horseradish peroxidase-conjugated anti-HFc antibody. Detection was performed using ECL.

will be of interest in testing whether Sk may be commonly up-regulated under such conditions.

There is evidence that members of the Wnt family play important roles in a variety of developmental processes (1, 2). Thus, it can be postulated that the simultaneous expression of Sk/Dkk-1 might be required to regulate Wnt function during development. According to their behavior following ectopic expression in *Xenopus* embryos and in mammalian cells, the Wnt genes have been divided into two functional classes (10, 34–36). The first, which includes Wnt-2, induces a secondary axis in *Xenopus* embryos, causes transformation of cells in culture, and signals through the β-catenin/T-cell factor pathway. The second class, which lacks these functions, is thought to activate independent signaling pathways. Thus, it will be of interest to assess whether Sk specifically antagonizes only those Wnts that signal through the β-catenin/T-cell factor pathway or interferes also with nontransforming Wnts.

The Sk/Dkk-1 proteins are not the only example of naturally occurring Wnt inhibitors. Like Sk/Dkk, members of the Frzb/FRP family are secreted by the gastrula organizer in *Xenopus*, have affinity for heparin, and are implicated in head formation (16, 18, 19). Frzb/FRP proteins contain a cysteine-rich domain, highly similar to the cysteine-rich domain present in the frizzled receptors, and appear to inhibit Wnt function by interfering with the frizzled/Wnt binding interaction (37). Another Wnt inhibitor is represented by the secreted protein Cerberus, which has been shown to bind and inhibit Wnts, among other proteins (21). More recently WIF-1, or Wnt inhibitory factor 1, has been described. This secreted protein, which contains five epidermal growth factor-like repeats in its sequence, was shown to inhibit axis induction by Wnts in *Xenopus* embryos (38).

The Sk/Dkk proteins contain two related cysteine-rich domains that are distinct from the FRP cysteine-rich domain but are highly conserved among members of the Dkk family. The mechanism by which Sk inhibits Wnt has not yet been elucidated. In the *Xenopus* embryo system, Dkk-1 and Frzb exhibit similar patterns of expression, and both antagonize Wnt signaling upstream of dishevelled, suggesting that both may act at an extracellular level (22). When compared with Frzb, Dkk-1 appeared to be a more potent head inducer and possibly a more effective Wnt antagonist (22). Consistent with these findings, when we compared the ability of Dkk-1 and FRP to interfere with Wnt signaling in NIH3T3 cells, Dkk-1 was a more potent Wnt inhibitor.[2] Further studies will be required to determine whether Sk/Dkk binds Wnt, like FRP/Frzb and Cerberus, or antagonizes Wnt signaling indirectly through an independent inhibitory pathway.

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